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ORIGINAL ARTICLE

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Arterial cells and CNS sheath cells from *Aplysia californica* produce factors that enhance neurite outgrowth in co-cultured neurons

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Abstract Substrate-bound and soluble factors regulate neurite outgrowth and synapse formation during development, regeneration, and learning and memory. We report that sheath cells from CNS connectives and arterial cells from the anterior aorta of the sea slug, *Aplysia californica*, enhance neurite outgrowth from co-cultured *Aplysia* neurons. Sheath and arterial cell cultures contain several cell types, including fibrocytes, myocytes, and amoebocytes. When compared to controls (neurons with defined growth medium alone), the percentage of neurons with growth and the average neurite lengths are significantly enhanced by sheath and arterial cells at 48 h after plating of the neurons; these parameters are comparable to those of neurons cultured in medium containing hemolymph. Our results indicate that sheath cells produce substrate-bound factor(s) and arterial cells produce diffusible factor(s) that promote growth. These growth factors likely promote neuron survival and neurite outgrowth during neural plasticity exhibited in the adult CNS.

Keywords Mollusk · Neurotrophic factors · Neurite outgrowth · Growth factors · Regeneration

Introduction

Neurotrophic factors modulate neuronal survival, neurite outgrowth, axonal pathfinding, and synapse formation (Henderson 1996; Huang and Reichardt 2001; Ibanez 1998). They mediate structural and functional changes in neurons and can act as tropic and/or trophic factors. For instance, nerve growth factor not only promotes survival and outgrowth of neurons from chick dorsal root ganglia, but can also attract the growth cones of dorsal root axons (Gundersen and Barrett 1979). Growth factors are derived from target tissues and cells in passage (for example, support cells or adaxonal glia; Sanes et al. 2000).

Developing and regenerating neurons are guided by short-range, substrate-bound factors (such as extracellular matrix molecules, ECMs, and cell adhesion molecules, CAMs) and long-range, diffusible factors (such as soluble growth factors). Both soluble and substrate-bound factors can either attract or repel growth cones (Fawcett and Asher 1999; Fournier and Strittmatter 2001; Tessier-Lavigne and Goodman 1996). In response to these factors, growth cones orient and turn as well as stop growing prior to synapse formation (Turner and Flier 1989). Molecules involved in axon growth and guidance are phylogenetically conserved from the level of lower invertebrates to mammals and, therefore, information derived from invertebrates is relevant to other species (McKay et al. 1999; Tessier-Lavigne and Goodman 1996).

Mollusks recover rapidly after lesions to the central nervous system (CNS) (Moffett 1995, 1996), and they present advantages for studies of CNS regeneration, including the presence of large, identifiable neurons (Kandel 1979; Kupfermann et al. 1991). When molluscan neurons are axotomized or transplanted, they can regenerate appropriate synaptic connections and show functional behavioral recovery (Benjamin and Allison 1987; Fredman 1988; Hamilton and Fredman 1998; Murphy 1990; Scott and Kirk 1992; Syed et al. 1992). In addition, behaviorally relevant components of molluscan neural circuits have been grown in tissue culture (Magoski and Bulloch 1999; Schacher 1988; Syed et al. 1990). Some evidence exists for support cells playing a role in the growth and differentiation of neurons during early development in the gastropod mollusk, *Aplysia californica* (Schacher 1981).

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Evidence exists for both unique invertebrate neurotrophic factors and invertebrate homologues of vertebrate neurotrophic factors (Liu et al. 1997; McKay et al. 1999). In addition, homologues of vertebrate ECMs and their integrin receptors as well as homologues of vertebrate CAMs are found in the nervous systems of invertebrates (Blumberg et al. 1987; Masuda-Nakagawa et al. 1993; Mayford et al. 1992). In fact, recent evidence suggests that integrins are involved in short-term memory in *Drosophila* (Grotewiel et al. 1998) and may be involved in long-term synaptic plasticity in *Aplysia* (McKay et al. 1999).

Examples of growth factors found in mollusks include a novel cysteine-rich neurotrophic factor and a molluscan epidermal growth factor isolated from the pond snail *Lymnaea* (Fainzilber et al. 1996; Hermann et al. 2000; Wildering et al. 2001). Recently, mollusk-derived growth factor was isolated and characterized in *Aplysia* (Akalal and Nagle 2001), and this growth factor may function during development and regeneration in adult animals. However, to date no endogenous receptors for molluscan growth factors have been purified and characterized. There are also numerous examples of functional effects produced in invertebrates in response to vertebrate growth factors (for a review, see McKay et al. 1999).

Recently, we have focused on functional CNS regeneration leading to recovery of consummatory feeding behavior in *Aplysia* (Johnson et al. 1999; Sánchez et al. 2000; Scott and Kirk 1992; Scott et al. 1995, 1997b). Rhythmic biting is eliminated by bilateral transections of the cerebral-buccal connectives (CBCs), without disrupting the appetitive phases of feeding behavior (Kupfermann 1974; Scott et al. 1995). Crush lesions applied to the CBCs initially eliminate rhythmic biting, but by 14 days after the lesions, biting behavior recovers (Scott and Kirk 1992; Scott et al. 1995). This behavioral recovery correlates with restoration of axonal projections in the CBCs and synaptic connections in the buccal ganglia, as demonstrated by ultrastructural and physiological investigations of regenerating neurons (Johnson et al. 1999; Sánchez et al. 2000; Scott et al. 1997b).

An important issue related to neural regeneration in the CBCs after a crush lesion is whether sheath cells surrounding the axonal core of the connectives, microglia, and/or adaxonal glia contribute to neurite growth and behavioral recovery after injury (von Bernhardi and Muller 1996; Johnson et al. 1999; Sánchez et al. 2000). Therefore, we initiated experiments to test for growth-promoting or inhibiting effects on co-cultured *Aplysia* neurons by sheath cells dissociated from central connectives of *Aplysia*. We also tested the effects on neurite outgrowth by cells dissociated from an unrelated tissue, the anterior aorta (AA), a potential source of neurotrophic factors present in the hemolymph of *Aplysia* (Schacher and Proshansky 1983; Srivatsan and Peretz 1997).

We describe here the characteristics of sheath cells from central connectives and arterial cells from the AA of *A. californica*, after dissociation of these cells and growth in tissue culture. The sheath and arterial cell cultures are comprised of myocytes, fibrocytes, and amoebocytes that display unique and complex morphologies. *Aplysia* neurons grown in the presence of the sheath or arterial cells exhibit enhanced neurite outgrowth. Evidence is presented that sheath cells primarily produce substrate-bound growth factor(s) while arterial cells likely produce diffusible factors that increase neurite outgrowth. Some of this work has appeared in abstract form (Marsh et al. 1999; Montgomery et al. 1998).

Materials and methods

Sheath cell and arterial cell cultures

We developed protocols for dissociating sheath cells and arterial cells from pleural-visceral connectives (PVCs) and a segment of the AA, respectively (Fig. 1). These protocols were based on methods used to dissociate buccal muscle fibers as described by Ram and Liu (1991) and Scott et al. (1997a) and modified for the PCVs and AAs as described here. Subjects (125–250 g *A. californica*; Marinus, Long Beach, Calif., USA) were first anesthetized by injecting a volume of isotonic $MgCl_2$ equal to half the animal's body weight. Using a ventral approach, the body cavity was exposed and the digestive tract pinned to one side to expose the PVCs and AA. We chose the PVCs for the present study because they are the longest connectives between central ganglia in *Aplysia*, they provide the greatest amount of starting material when compared to other central connectives, and the connective tissue of all sheaths surrounding central neurons in *Aplysia* appears to be comprised of similar cell types (Coggeshall 1967). The entire length of both PVCs and a 2-cm section of the AA starting just anterior to emergence of the dorsal artery, were removed. The AA was used because of its large size, its known composition (including myocytes), and the fact that it may be a potential source of growth factors present in hemolymph (Schacher and Proshansky 1983).

To avoid contamination by unrelated cell types, adherent mesenteric tissue was cleared from the PVCs and AA. The PVCs and AA were then placed in 1.5 ml Eppendorf tubes containing 100 µl collagenase (C-8176; Sigma, St. Louis, Mo., USA) brought to a final volume of 1 ml using supplemental Leboweitz-15 medium (SL-15; L-4386; Sigma; Schacher and Proshansky 1983). The tissues were incubated in a 34°C water bath for 45 min to 1 h, with gentle agitation. Starting at 20 min the tissues were checked visually every 5 min to assess the extent of tissue digestion, and once the PVCs or AA acquired a feathered or ragged appearance and cells appeared in the solution, rinsing and trituration were initiated.

To rinse the tissues they were pelleted (14,000 rpm, 60 s), the collagenase solution removed, and the tissue resuspended in 1.5 ml SL-15. Rinsing was repeated three times. After the final resuspension in SL-15, trituration was completed by pipetting 800 µl of the tissue suspension repeatedly (approximately 40 times) in the same tube using a blue tip (pipette tip, vol = 1 ml). Then 750 µl of dispersed PVC cells were plated evenly across a glass coverslip; the latter had been previously placed in culture dishes with tight-fitting lids and coated with poly-L-lysine (P-1524; Sigma; Goldberg and Schacher 1998). The dissociated PVC/AA cells were then cultured up to a total of 48 h at 18°C before fixation, staining, and photography. Alternatively, dispersed neurons were added to the cultures the morning after the PVC/AA cells were plated.

Neuron co-cultures

Neurons were dissociated from the pleural and pedal ganglia of 10–25 g *A. californica*, obtained from the National Resource Facility for *Aplysia* at the University of Miami. The cultures of dispersed neurons were performed according to published

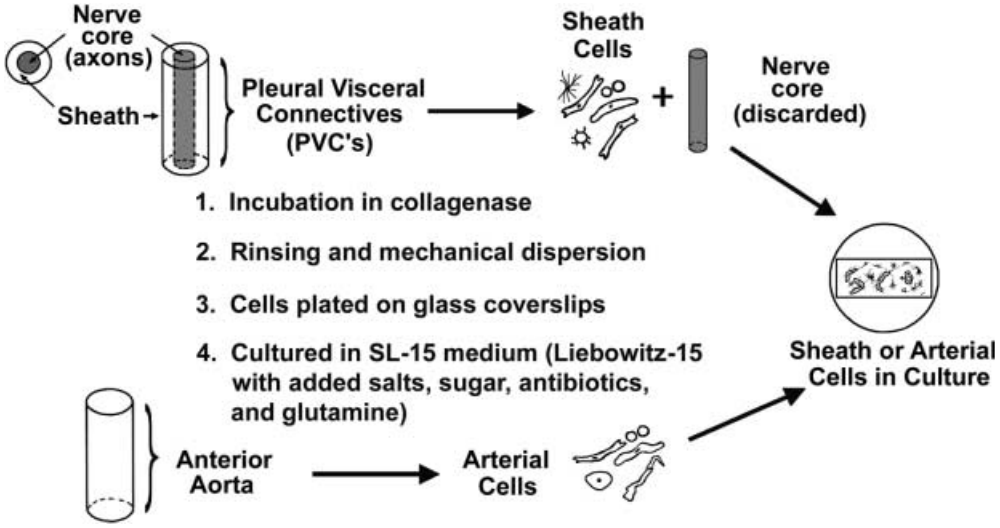

Fig. 1 Sheath and arterial cell cultures were established as shown schematically. The entire length of both pleural-visceral connectives (PVCs) and a 2-cm section of anterior aorta (AA) were dissociated by a combination of enzymatic treatment and mechanical trituration. Subsequently, the dissociated cells from PVCs or AA were plated on separate glass coverslips coated with poly-L-lysine and cultured in supplemental Leboweitz-15 medium (SL-15; Schacher and Proshansky 1983). For neural co-cultures, neurons were dispersed from pleural-pedal ganglia as described previously (Goldberg and Schacher 1998) and plated on top of either sheath or arterial cell cultures. The latter was allowed to sit overnight at room temperature. Neural co-cultures were incubated at 18°C for up to 48 h before fixation and labeling

procedures (Goldberg and Schacher 1998). Briefly, two pleural and two pedal ganglia were removed from anesthetized animals (see above) and placed in 0.55% w/v protease (type IX; P-6141; Sigma) in SL-15 and allowed to incubate from 40 min to 1.5 h at 34°C with gentle agitation. The extent of digestion was checked every 10 min starting at 40 min, and the digestion stopped when the sheath could be pulled away easily with forceps and the neurons appeared firm, with distinct outlines. The ganglia were then rinsed three times in SL-15 and placed in an 18°C incubator to rest for 1–2 h. The treated ganglia were transferred to a Sylgard-lined glass petri dish and surgically desheathed. After desheathing, larger ganglia were cut into two to four small pieces. Ganglionic clusters or whole ganglia were transferred into a 1.5-ml Eppendorf tube using a pipette tip (''blue tip'') whose opening had been slightly enlarged by cutting it at an angle. To acquire dispersed neurons, the ganglia and ganglionic clusters were mechanically triturated using pipette tips (200 µl capacity ''yellow tips'') with openings that had been slightly enlarged and fire-polished to make smooth. One milliliter of SL-15 containing the neural suspension was expelled into an appropriate culture dish (including on top of previously cultured PVC/AA cells, see above) to evenly disperse the neurons onto the coverslip. In all experiments the neurons were plated throughout the coverslips and at a low density to prevent contact with each other after 48 h of growth (neurons contiguous with adjacent neurons were not included in the present studies). Fifteen minutes after plating the neurons, 3 ml SL-15 was added to the culture plate, and the plates were allowed to sit at room temperature overnight before incubating at 18°C for up to a total of 48 h. Negative controls consisted of dispersed neurons cultured in SL-15 alone, whilst positive controls contained dispersed neurons cultured in an equal mixture of SL-15 and hemolymph (50:50).

To test for the presence of diffusible factors, the PVC/AA cells were plated on only one half of the coverslip and neurons were dispersed throughout the coverslip as described above. In this case, 325 µl of the triturated and rinsed PVC/AA cell suspension was added evenly over half of the coverslip surface. The plated PVC/AA cells were allowed to settle and attach to the coverslip before 2 ml SL-15 was added, and they were cultured overnight at room temperature before adding the neurons. Using this procedure, most if not all of the PVC/AA cells were subsequently found on only one half of the coverslip with no PVC/AA cells observed on the other half of the coverslip. Inevitably, some debris and support cells were present in the aliquots of dispersed neurons, and the former were found on the side of the coverslips not containing PVC/AA cells. However, debris and support cells (for example, adsomatic glia; Lockhart et al. 1996) present in our aliquots of dispersed neurons had no overt effect on neurite growth in control conditions (i.e., SL-15 alone; see Results and Discussion). We also tested for the presence of diffusible factors in sheath cell–neuron co-cultures by gently agitating the culture dishes. To facilitate mixing of factors within the culture medium, we placed the culture dishes on a slowly rocking platform for the duration of their incubation.

Immunocytochemistry and microscopy

Preparation of cells for immunocytochemistry and fluorescence or phase-contrast microscopy was accomplished as follows. Cells were fixed in 4% paraformaldehyde, 0.1 M phosphate-buffered saline (PBS), and 0.3 M sucrose, and permeabilized using 0.1% Triton X-100 in 0.1 M PBS for 45 min. Subsequently the cells were rinsed three times in 0.1 M PBS.

An effective method to determine the morphology of sheath cells and arterial cells was to label them with phalloidin tagged with Alexa Fluor 594 or Oregon green 488 (A-12381 and O-7466, respectively; Molecular Probes, Eugene, Ore., USA) as phalloidin binds F-actin. Permeabilized cells were exposed to phalloidin (diluted 1:1,000 in 0.1 M PBS) overnight at 4°C. Neurons were labeled using a monoclonal antibody to β-tubulin (E7; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA) and a goat anti-mouse secondary antibody conjugated to Texas Red (Jackson Immunoresearch Laboratories, West Grove, Pa., USA). In some experiments, nuclei of cultured cells were labeled with Hoechst 33258 (B-2883; Sigma). The Hoechst stain was diluted in 0.1 M PBS (5 mg/ml) and applied to the coverslips either for 1 h at room temperature or overnight at 4°C. After labeling, cultures were rinsed in 0.1 M PBS prior to viewing and photography.

For image capture, cultured sheath, arterial, and neural cells were viewed using phase contrast, Nomarski, and florescence microscopy. Images were acquired for color slides using an Olympus BH2 epifluorescence microscope (Ektachrome 400 ASA slide film). Digital images were captured using two systems. The first system used was an inverted Olympus IX70 microscope equipped with Uniblitz shutters, a 3-CCD video camera (Optronics DEI-750TD), and a G4 Mac OS computer equipped with the OpenLab software package (Improvision). The second system for digital image

capture also used an Olympus IX70 but with a Photometrics Sensys CCD camera. The software for the latter system was Image Pro Plus Version 4.1 by Media Cybernetics (8484 Georgia Avenue, Silver Spring, Md., USA) Within Image Pro, Vay Tek Volume Scan Version 3.1 by Vay Tek, was used for image capture. For differential interference contrast (DIC, Nomarski optics) images, a condenser polarizer, Olympus U-DICT Nomarski Prism, and an Olympus IX-AN Analyzer were used. Because the microscope systems used for digital image capture possessed epifluorescence, phase-contrast, and Nomarski optics capabilities, immunofluorescent images of labeled cells as well as phase-contrast and/or Nomarski images of fixed and unstained neurons were obtained.

Measurements and statistics

Measurements of percentage of cells with growth, soma diameters, and neurite lengths were made under phase-contrast conditions using a Nikon Diaphot inverted microscope with an ocular micrometer. In some cases, digital images were captured as described above and saved as TIFF files for subsequent analysis using NIH Image. The number of cells sampled for each parameter is indicated in the text or figure legend, and the minimum number of co-culture preparations or control cultures obtained for each experiment (n) was five.

The number of neurites was measured by counting primary neurites, and a primary neurite was defined as a neurite originating directly from the soma (or from one main neurite, if present after isolation; see Results). Neurite length was measured by incremental measurements along the length of primary neurites to their distal endings, the latter usually consisting of a prominent growth cone. Neurons were considered to have growth if they exhibited at least one primary neurite and it had acquired a length greater than that of one soma diameter.

To obtain unbiased samples of the variables outlined above, a physical template whose perimeter matched the outer dimensions of the coverslips was constructed. The template consisted of three 1-cm^2 openings, evenly spaced across the length of the template. The template was visually centered over the coverslip (from the underside of the petri dish), the three square areas were marked on the bottom of the culture dish, and only neurons found within these sample areas were used for measurements.

Data were analyzed using SAS with assistance by the University of Missouri-Columbia Statistical Consulting Service. For some results, SPSS was used. The number of neurons quantified for a given category of experiment ranged from 31 to 546. Data are presented as means ± standard errors. An independent-samples t-test was performed when comparing the dimensions of myocytes, and unless otherwise indicated, analysis of variance (ANOVA) was performed in all other cases. Significance was established at the $P < 0.01$ level unless otherwise indicated.

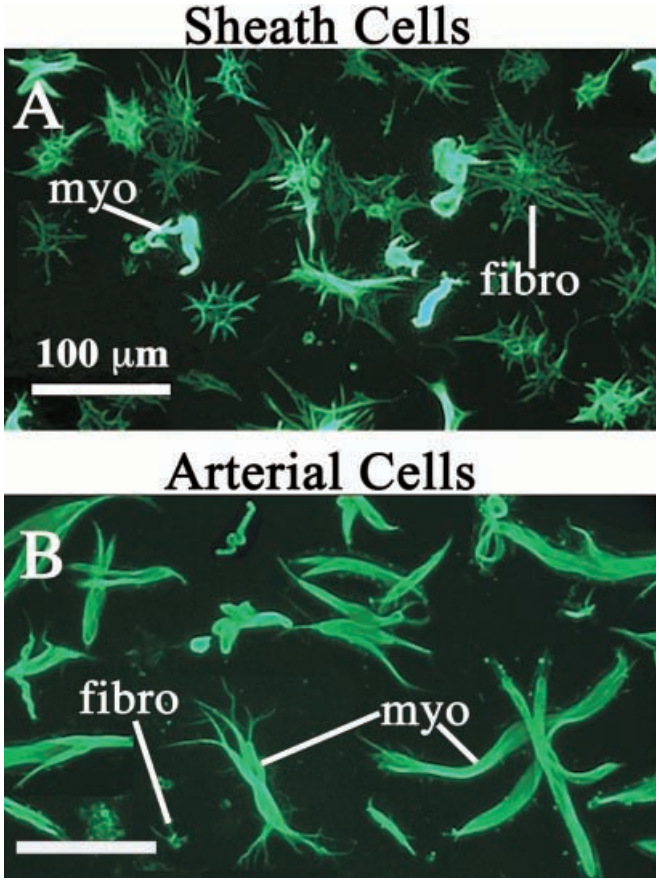

Fig. 2A, B Sheath and arterial cell cultures were complex in nature, containing at least three cell types. **A** The most prominent cell types in sheath cell cultures were myocytes (*myo*), elongated, thin cells with numerous filopodia and intense labeling for F-actin, and fibrocytes (*fibro*), cells that were flat and spread out, with numerous bundles of F-actin evident in their cytoplasm. **B** The arterial cultures were dominated by large myocytes (*myo*), long cells with large diameters, some exhibiting a branched morphology. An occasional fibrocyte (*fibro*) was also observed in cultures of AA. Amoebocytes were smaller, labeled less intensely for F-actin and were not clearly evident in these low-power images (see Figs. 3, 4). In **A** and **B**, the cultured cells were labeled with phalloidin–Oregon green (phalloidin binds F-actin). Calibration in **A** applies to **B**

Results

Dispersed sheath cell and arterial cell cultures

Primary cultures of dissociated AA or PVCs were obtained and plated on glass coverslips coated with poly-L-lysine. These cultures were complex in nature and contained several cell types. The morphology of the dispersed cells was revealed in some detail after labeling for F-actin (Figs. 2, 3, 4) and/or tubulin (Figs. 4I, 5E, F).

Cellular components of sheath cell cultures

Sheath cell cultures largely contained myocytes, fibrocytes, and amoebocytes (Figs. 2A, 3). Figure 2A illustrates a typical sheath cell culture at low power labeled with phalloidin–Oregon green and demonstrates that myocytes and fibrocytes were the largest and most prominent cell types in these cultures. This was confirmed with labeling for nuclei using Hoechst dye (data not shown), and with Nomarski optics (data not shown). Fibrocytes appeared as flattened cells that extended multiple processes from their perimeter and contained distinct bundles of actin within their cytoskeleton (Figs. 2A, 3A, C–F).

Myocytes were elongated cells, usually extended numerous processes along their length, and showed intense labeling for actin throughout the cytoplasm (Figs. 2A, 3A–C). On occasion myocytes were observed to have contracted prior to or during fixation, and appeared as large, oval-shaped cells exhibiting intense phalloidin labeling (data not shown). Myocytes dissociated from

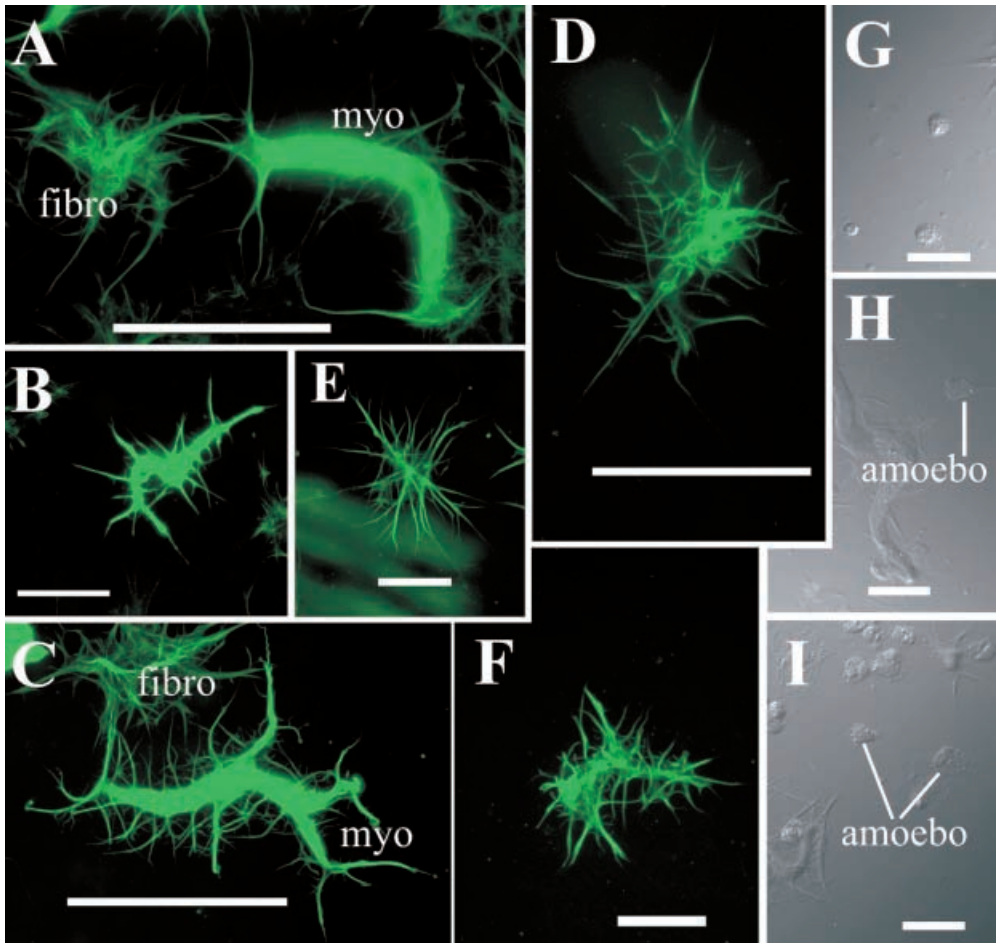

Fig. 3A–I Sheath cell cultures were composed of myocytes (*myo*), fibrocytes (*fibro*), and amoebocytes (*amoebo*). **A–C** Myocytes were long, narrow cells that exhibited intense staining for cytoplasmic F-actin and displayed numerous processes. **D–F** Fibrocytes appeared flattened and contained distinct bundles of F-actin within their cytoplasm (examples of fibrocytes are also shown in **A** and **C**). Myocytes and fibrocytes shown in **A–F** were labeled with phalloidin–Oregon green. **G–I** Amoebocytes (shown here in Nomarski images) were spherical, exhibited veils of membrane surrounding the nucleus, and occasionally displayed processes that appeared to emerge from the membranous veils. Calibrations: **A–C** 100 μm; **D–F** 50 μm; **G–I** 25 μm

PVCs were small in length (mean = 135.5 ± 10.4 μm, $n = 16$) and width (mean = 8.1 ± 0.3 μm, $n = 16$) and were significantly smaller than myocytes obtained from AA (see below).

Amoebocytes were identified as spherical cells that often exhibited veils of membrane and/or limited process outgrowth (Fig. 3G–I). Amoebocytes appeared to be less abundant in these cultures than myocytes and fibrocytes, although this observation may have resulted from a less intense labeling of amoebocytes with markers for F-actin. It is likely that cell types other than those described above were present in sheath cell cultures, as we observed small cells labeled with phalloidin that could not be unambiguously categorized (data not shown).

Cellular components of arterial cell cultures

Figure 2B illustrates a typical culture of dissociated AA cells. Similar to sheath cell cultures, at least three cell types were present in AA cultures, including myocytes, fibrocytes, and amoebocytes (Fig. 4). The most striking feature of the AA cultures was the presence of large, branched myocytes that often exhibited filiform processes extending from the perimeter of the myocytes (Figs. 2B, 4A–D). The mean width and mean length (22.4 ± 1.6 and 297.1 ± 48.2 μm, $n = 16$ cells, respectively) of AA myocytes were significantly greater ($P < 0.0001$ for both width comparisons and length comparisons) than those of sheath myocytes. Myocytes dissociated from PVCs and AA were mononucleate, as revealed by labeling with Hoechst dye (data not shown).

Amoebocytes and fibrocytes were also observed in the AA cultures, and they exhibited morphologies somewhat similar to those of amoebocytes and fibrocytes observed in sheath cell cultures (Fig. 4E–K). That is, fibrocytes were flattened and contained distinct bundles of cytoskeletal actin, and amoebocytes appeared rounded, contained discrete cytoplasmic granules when viewed with Nomarski optics, and usually extended few processes from their perimeter (Fig. 4E, H–K). However, the fibrocytes in AA cultures were more diverse in their morphologies than fibrocytes derived from PVCs. Fibrocytes from AA exhibited a wide range of shapes and variable patterns of F-actin bundling within their cytoplasm (Fig. 4E, F). We also observed clustering of amoebocytes in AA cultures (Fig. 4J), although not in contact with the neurites of co-cultured neurons (Fig. 5F). As with the sheath cell cultures, we observed smaller cell types of unknown nature.

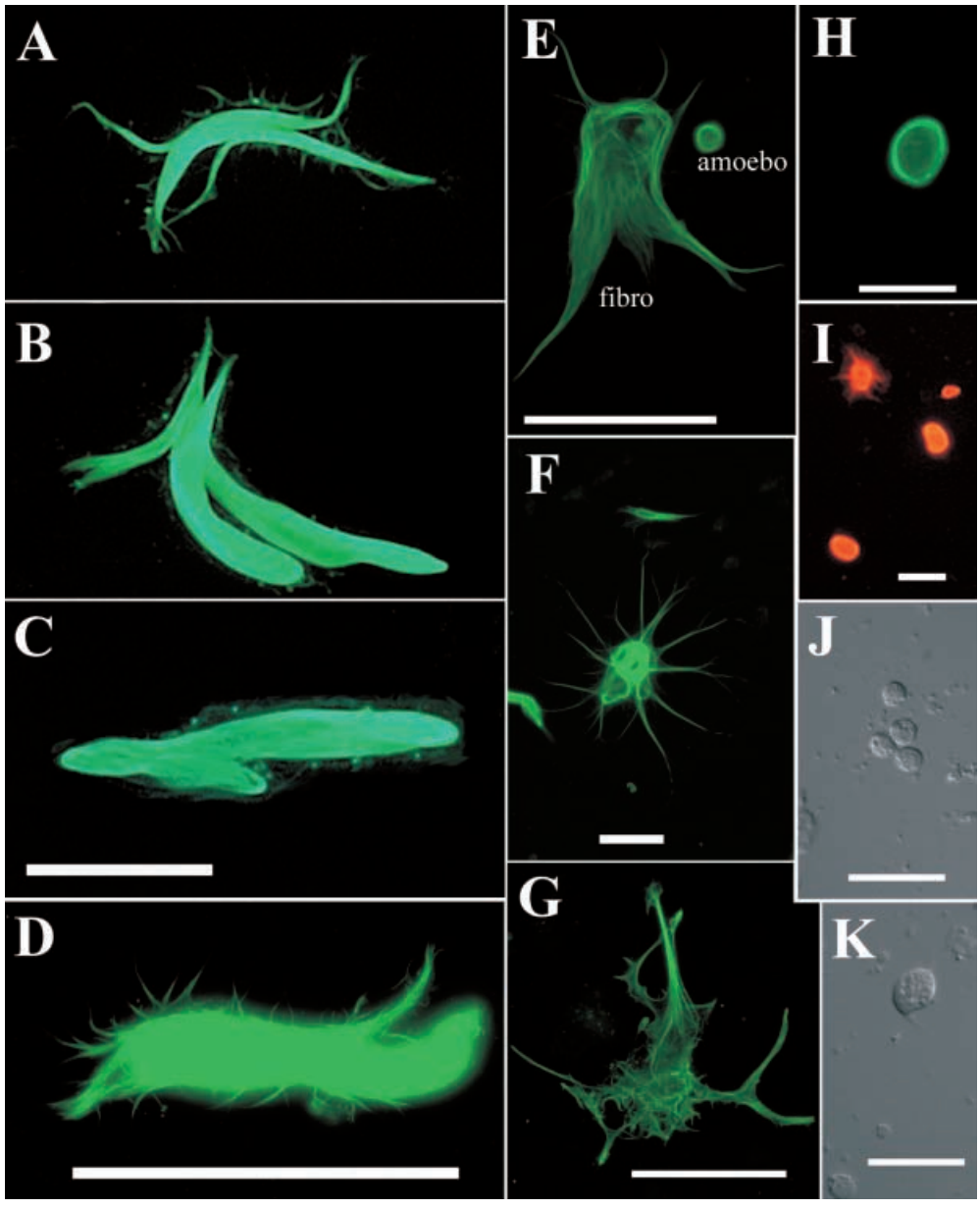

Fig. 4A–K Arterial cell cultures were dominated by large myocytes and also contained smaller fibrocytes (*fibro*) and amoebocytes (*amoebo*). **A–D** The myocytes were often branched and in most cases exhibited numerous filipodia projecting from their plasma membrane. **E–G** Fibrocytes found in arterial cultures assumed varied morphologies (more varied than in cultures of PVC sheath cells). All fibrocytes displayed F-actin bundles within their cytoplasm and exhibited process outgrowth. **H–K** Amoebocytes were spherical, occasionally exhibited membranous veils (**I**), and contained distinct cytoplasmic granules; the latter were most clearly observed using Nomarski optics (**J**, **K**). Some clustering of amoebocytes was observed (**J**). An example of an amoebocyte is also shown in **E**. Preparations shown in **A–H** were labeled with phalloidin–Oregon green; in **I** the preparation was labeled with a primary antibody for β-tubulin and a secondary antibody conjugated to Texas red; **J** and **K** are Nomarski images. Calibrations: **A–D** 100 μm; **E–G** 50 μm; **H–K** 25 μm

Neurotrophic actions of sheath and arterial cells

Factors produced by dissociated sheath and AA cells had neurotrophic effects on co-cultured *Aplysia* neurons. Neurons were dispersed at low density throughout coverslips containing either sheath or AA cells (Figs. 5, 6) and allowed to grow for up to 48 h. Measurements were obtained at 12, 24, 36, and 48 h after plating the neurons, and growth in all conditions was compared to that observed in SL-15 alone. The latter is a minimal essential medium (with added salts) used to maintain *Aplysia* neurons in culture, and was the only medium used in all experiments, except in cultures containing hemolymph.

The SL-15 medium alone supported a small amount of growth by pleural/pedal neurons (Fig. 5A). We observed an increase in the number and length of primary neurites by factors produced by sheath and AA cells (see below). However, there was no overt effect on the general morphology of the cell bodies or growth cones. As previously reported (Schacher and Proshansky 1983), we observed two main types of neurite branching patterns from isolated neurons, depending on the presence or absence of a main neurite attached to the cell body after trituration. First, if no main neurite was present after plating the soma, we observed growth of multiple primary neurites from cell bodies (Fig. 5B–D). Alternatively, if a substantial portion of the main neurite remained attached to the cell body, most subsequent neurite growth occurred from the distal end of the main neurite (Fig. 5A, E, F). In the present study, processes emerging from the main neurite were defined as primary neurites, and if there was no main neurite attached after isolation, all processes emerging from the soma were considered to be primary neurites. As described below,

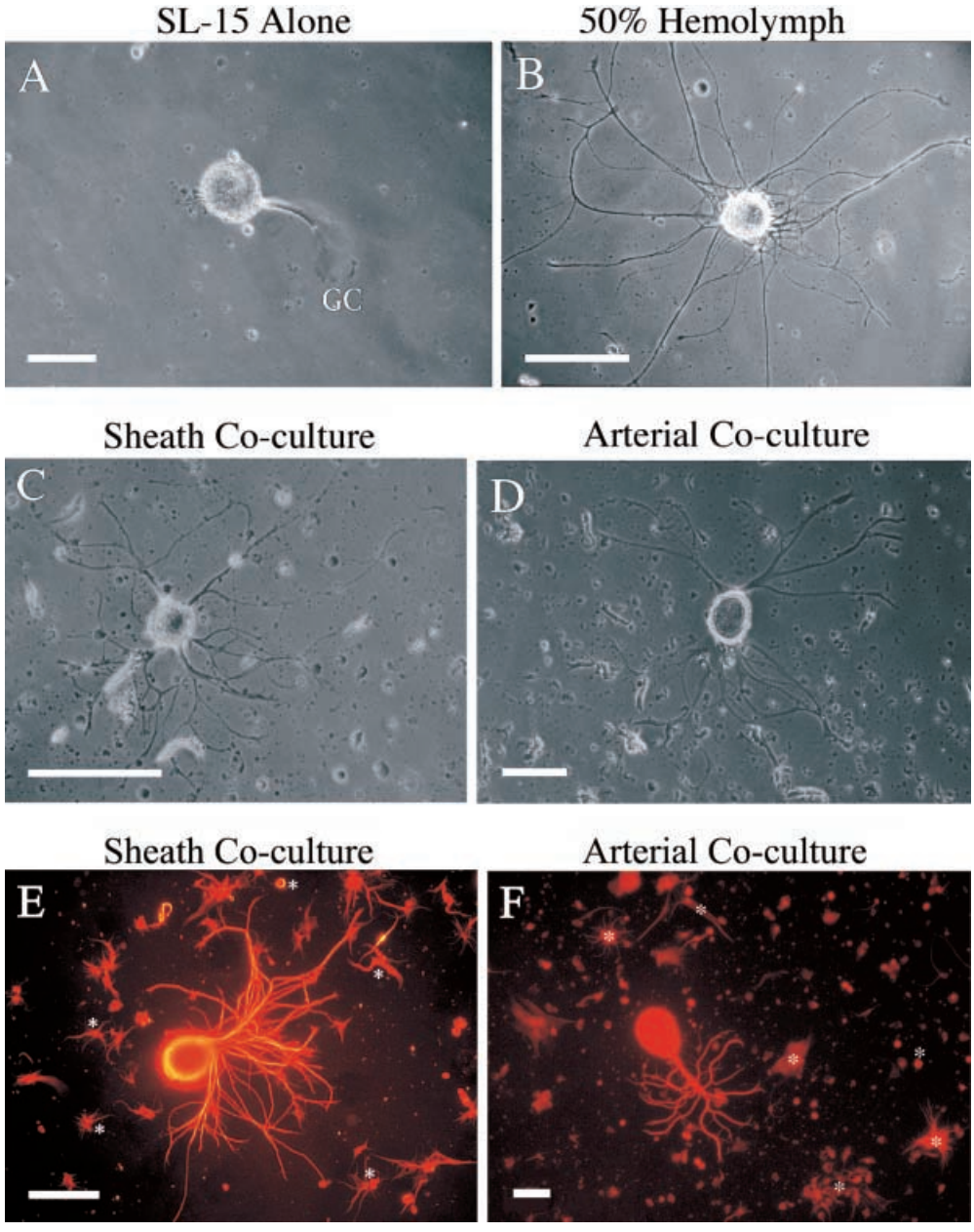

Fig. 5A–F Factors synthesized by sheath and arterial cells had neurotrophic effects on co-cultured neurons. **A** Under negative control conditions (medium consisting of SL-15 alone), a very limited amount of neurite growth was exhibited by cultured pleural/pedal neurons. *GC* Growth cone. **B** In positive control conditions (medium consisting of equal amounts of SL-15 and hemolymph; 50% hemolymph), extensive neurite outgrowth was observed. **C**, **D** The presence of sheath and arterial cells in the neural co-cultures greatly enhanced neurite outgrowth. Note that the neurons shown were lacking a main neurite after trituration, and multiple primary neurites regenerated from the cell bodies. **A–D** Phase-contrast images. **E**, **F** When a main neurite remained attached after trituration, most of the neurite regeneration in the presence of sheath (**E**) or arterial (**F**) cells occurred from the distal end of the remaining main neurite. In **E** and **F** preparations were labeled with a primary antibody to β-tubulin and a secondary antibody conjugated to Texas red. *Asterisks* indicate examples of isolated sheath or arterial cells. In **F** note the *lowest asterisk* in the *lower right corner* tags a cluster of amoebocytes. All calibration bars in this figure are 100 μm

the percentage of cells with growth, number of primary neurites, and average primary neurite length varied with the culture conditions.

Sheath and arterial cell factors increase the percentage of cells with growth

Sheath and AA cells significantly enhanced the percentage of neurons with growth, when compared to cultures containing SL-15 alone (Figs. 5A, C–F, 6A). A significant increase in percentage of neurons with growth, between the SL-15 and sheath cell conditions, was observed as early as 24 h after plating the neurons. The percentage of neurons with growth increased at 36 and 48 h post-plating. In fact by 48 h, the percentage of neurons with growth observed in the presence of sheath cells was comparable to that observed when the culture medium contained *Aplysia* hemolymph (SL-15: hemolymph, 50:50; Figs. 5B, C, 6A). Culture medium containing 50% hemolymph strongly promotes growth by *Aplysia* neurons (Burmeister et al. 1991; Goldberg and Schacher 1998; Schacher 1988; Schacher and Proshansky 1983). Culture medium consisting of SL-15 alone was sufficient to sustain minimal growth; however, no significant change in the percentage of neurons with growth occurred in this condition throughout the 48-h culture period (logistic regression analysis, $P < 0.01$).

When neurons were plated in the presence of AA cells, there was a significant increase in percentage of neurons with growth at all times post-plating, compared to SL-15 alone (Fig. 6A). By 48 h the percentage of

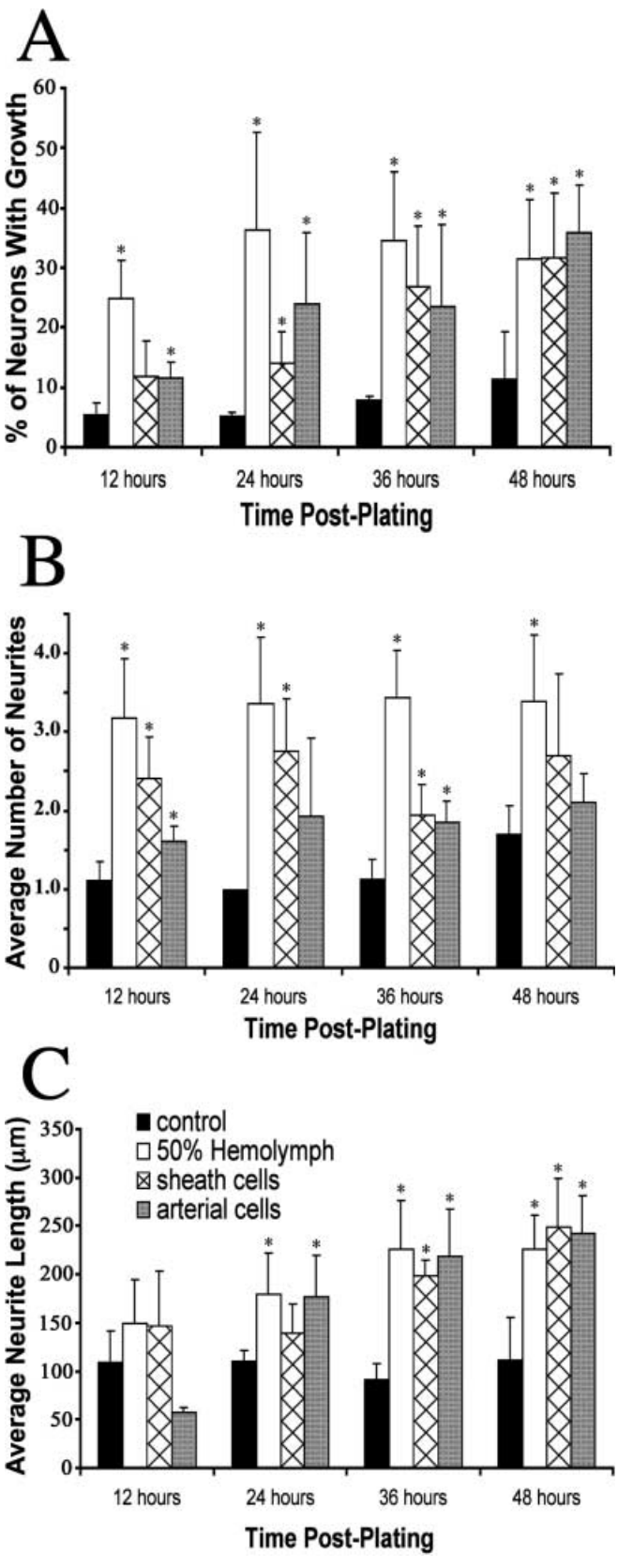

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Fig. 6A–C Sheath and arterial cells significantly increase the percentage of neurons with growth, their average number of neurites, and the average length of neurites growing from a neuron. **A** The percentage of neurons with growth increased in the presence of sheath or arterial cells. A significant effect (when compared to SL-15 alone) was observed for both sheath and arterial cell cultures by 24 h after plating the neurons (i.e., *time post-plating*), and the enhancement of growth continued to increase through 48 h, at which time it was comparable to that of positive controls (50:50, SL-15:hemolymph). **B** The average number of neurites per neuron increased in the presence of sheath and arterial cells. Although the number of neurites per neuron remained increased throughout the times tested for both sheath and arterial co-cultures, it was not significantly different from controls by 48 h after plating the neurons. **C** At 36 and 48 h post-plating, the average neurite length per neuron was significantly greater in the presence of sheath or arterial cells. All statistical comparisons in this figure were made using ANOVAs with *post hoc* LSD. *Asterisks* indicate a significant difference ($P < 0.01$) with respect to controls; controls consisted of neurons grown in culture medium containing SL-15 alone

neurons with growth was comparable to that of neurons plated in the presence of sheath cells or hemolymph. Although sheath and arterial cells both promoted neurite outgrowth from co-cultured neurons, there was no selective attraction to a particular cell type in either co-culture; that is, neurites did not grow selectively toward or accumulate near a specific sheath or arterial cell type (Fig. 5). The above results indicated that cultures containing hemolymph, sheath cells, or arterial cells all contained factors that enhance the growth of neurons.

Sheath and arterial cell factors increase the number of primary neurites and neurite length

The average number of primary neurites extending from isolated somata was increased in the presence of sheath cells, arterial cells, and hemolymph, when compared to SL-15 alone (Fig. 6B). This increase was significant for sheath cell co-cultures at 12, 24, and 36 h, for arterial cell cultures at 12 and 36 h, and for hemolymph at all times post-plating. By 48 h post-plating, the average number of primary neurites was still greater in the presence of sheath and arterial cells, but the differences were no longer significant with respect to that of SL-15 alone. The latter result was likely due to an increase in the number of neurites sprouting from somata in SL-15 alone at 48 h and to a plateau in the number of primary neurites produced in the presence of sheath and arterial cells (Fig. 6B). Note that in the presence of hemolymph, the average number of primary neurites that emerge from somata plateaus within the first 12 h of co-culture.

The most dramatic growth effect brought about by the presence of sheath and arterial cells was on the length of neurites with time post-plating of the neurons. By 24 h, the average neurite length in sheath and arterial cultures was greater than that observed in cultures with SL-15 alone (Fig. 6C). At 36 h, the average neurite length in the presence of sheath and arterial cells was twice that for neurons grown in SL-15 alone. At 36 and 48 h, sheath cell and arterial cell cultures exhibited neurite growth comparable to that produced by medium containing hemolymph, and at these times post-plating

the differences in average neurite length in all three experimental conditions were significantly greater than that observed in SL-15 (Fig. 6C).

Sheath cells produce substrate-bound factor(s) and arterial cells produce diffusible factor(s) that promote neurite growth

The neurotrophic responses elicited by sheath and arterial cells could result from production of one or more diffusible factors, transmembrane factors, and/or substrate-bound factors, such as ECMs. To test whether sheath and arterial cells produced diffusible factors, we performed the following experiments. Sheath or arterial cells were plated on one half of the coverslips, and neurons were subsequently dispersed throughout the coverslips. Therefore, neurons were cultured in regions of the coverslips with or without sheath or arterial cells.

Neurons grown in contiguity with sheath cells demonstrated significantly enhanced neurite growth when compared to SL-15 controls at all times post-plating (Figs. 7A, 8A; with the exception of average neurite length at 12 h post-plating), typical of neurons grown on coverslips in which sheath cells were plated on the entire surface of the coverslip (Fig. 5C, E). In contrast, neurons grown on the side of coverslips lacking sheath cells exhibited very limited growth (Figs. 7B, 8A), similar to neurite growth in SL-15 alone (Fig. 5A). The percentage of cells showing growth and average neurite length, for neurons grown on the side of the coverslip without sheath, were not significantly different from controls (SL-15 alone) at all times post-plating (Fig. 8). Note that in sheath/neuron co-cultures, amoebocytes occasionally aggregated around growing neurites (Fig. 7A).

In separate experiments, to enhance diffusion of factors in the culture medium we gently rocked the co-cultures for the duration of their incubation. The increased mixing of the culture medium had no overt effect on neurite outgrowth, including neurons in the region of the coverslip lacking sheath cells ($n = 3$, data not shown). Therefore, it appeared likely that diffusible growth factors that increased neurite growth were not present in the sheath cultures, and if a diffusible factor was produced, it was in limiting amounts so as to affect only neurons contiguous with sheath cells. These results suggested that sheath cells possessed at least one growth factor on their outer membranes and/or produced a substrate-bound factor that enhanced neurite growth.

In contrast to sheath cells, arterial cells plated on only one half of the coverslips increased neurite growth from neurons located throughout the coverslips. The percentage of cells showing growth was significantly different from controls 24, 36, and 48 h post-plating, regardless of their location on the coverslip (i.e., arterial or no arterial; Figs. 9, 10A).

Average neurite length also increased in response to factors produced by arterial cells, and the enhanced growth was observed throughout the coverslip. However, average neurite length was only significantly different from controls (SL-15 alone) at 48 h for neurons in areas of the coverslip without arterial cells. When contiguous with arterial cells, neurite length was significantly enhanced with respect to controls at 24 and 48 h (Fig. 10B), typical of neurons grown on coverslips in which AA cells were plated on the entire surface of the coverslip (Fig. 5D, F). These results are consistent with the production of diffusible neurotrophic factor by arterial cells.

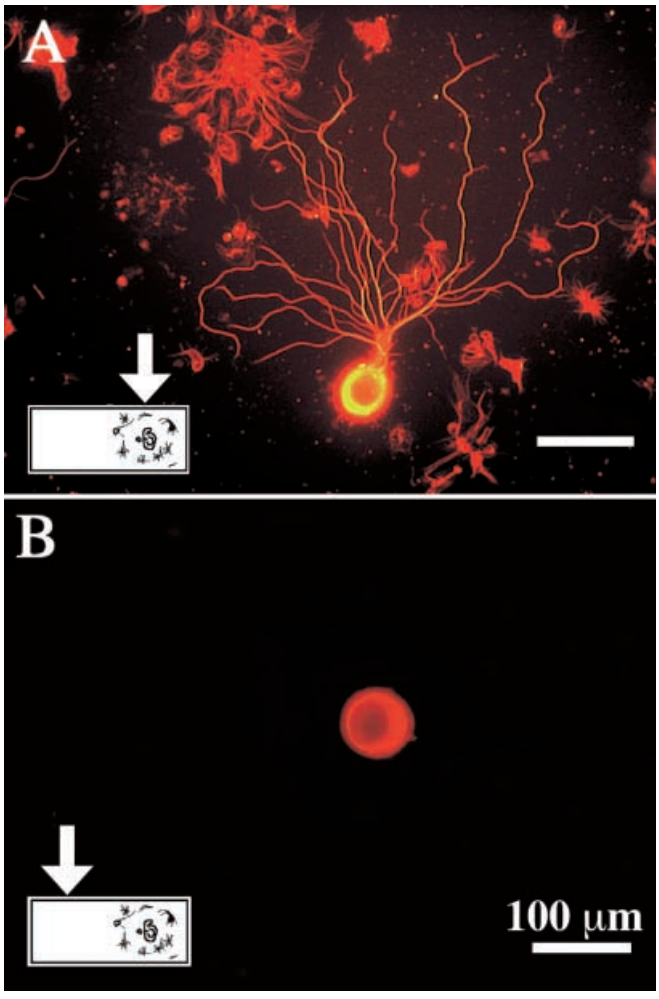

Fig. 7A, B Sheath cells produce one or more substrate-bound factors that enhance neurite growth. Sheath cells were plated on one half of the coverslip while neurons were distributed throughout the coverslip. **A** Neurons grown on the side of the coverslip with cells dispersed from the PVCs (relative location is indicated by the *inset*) exhibited extensive neurite outgrowth. Note the aggregate of amoebocytes (*upper left*) adjacent to neurite tips of the co-cultured neuron in the field of view. **B** Neurons grown on the half of the coverslip lacking sheath cells (see *inset*) exhibited limited growth, resembling neurons grown in culture medium containing SL-15 alone. Calibration in **B** applies to **A**

Discussion

Our results indicate that sheath cells dissociated from connectives of the *Aplysia* CNS produce at least one

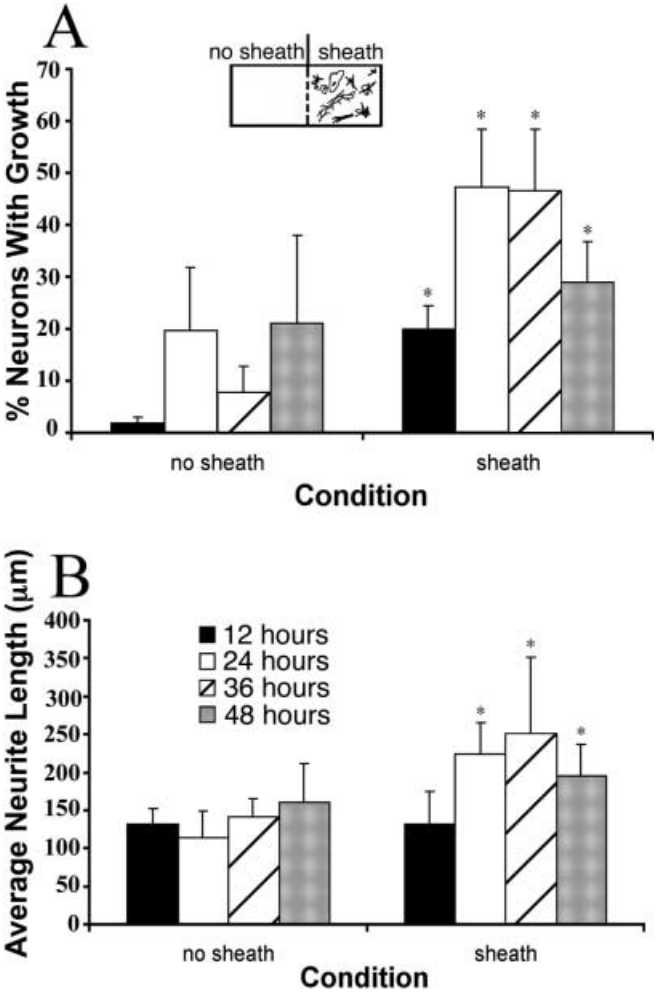

Fig. 8 Growth factor(s) produced by sheath cells increased the percentage of neurons with growth (**A**) and the average neurite length per neuron (**B**), but only when the neurons were contiguous with co-cultured sheath cells. At all times post-plating of the neurons and on the side of the coverslip containing sheath cells (sheath, see *inset*), the percentage of neurons with growth was significantly greater than for controls (i.e., neurons cultured with SL-15 medium alone). The average neurite length per neuron was significantly greater than controls for neurons plated on top of sheath cells, for 24, 36, and 48 h. *Asterisks* indicate significant differences from controls ($P < 0.01$)

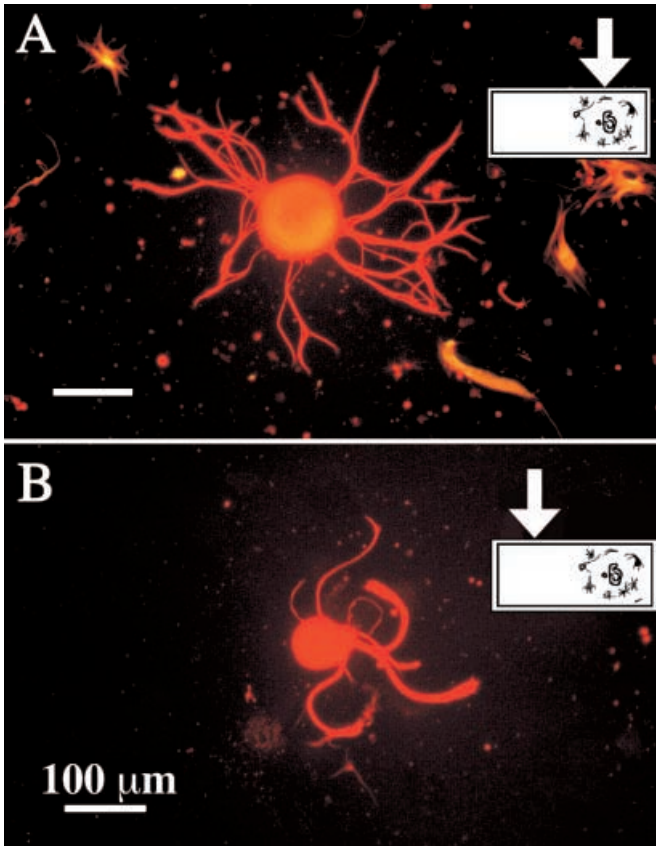

Fig. 9A, B Arterial cells produced a growth factor(s) that increased neurite growth by neurons throughout coverslips containing AA cells. **A** Neurons plated on top of co-cultured, dispersed AA cells (see *inset*) exhibited extensive neurite outgrowth. **B** Neurons plated on the side of the coverslip lacking arterial cells (see *inset*) also exhibited enhanced neurite outgrowth when compared to controls at 48 h (see Fig. 10). Calibration in **B** applies to **A**

substrate-bound factor that enhances neurite outgrowth from co-cultured neurons. Arterial cells dissociated from the AA produce at least one diffusible growth factor that increases neurite growth. If an inhibitory factor was produced by either sheath or arterial cells, inhibition of neurite outgrowth is overwhelmed by factors that enhanced neurite outgrowth.

The sheath and arterial cell cultures are complex, consisting of at least myocytes, fibrocytes, and amoebocytes; the latter may include hemocytes in arterial cultures and in the case of sheath cell cultures, microglia, hemocytes, and/or adaxonal glia. In leeches and mollusks (Bale et al. 2001; von Bernhardi and Muller 1996; Moffett 1995), amoebocytes (presumably microglia) are attracted to sites of nerve injury and appear to produce growth factors (for example, laminin in the case of leech; Masuda-Nakagawa et al. 1993) that enhance axonal regeneration. It is possible that adaxonal glia from axons located in peripheral regions of the axonal core of the PVCs are derived during the dissociation process, and may be present in our sheath cell cultures.

Hemocytes isolated from the hemolymph of *Aplysia* can avoid co-cultured neurons or can aggregate around and form stable contacts with the neurons (Farr et al. 2001). In some cases, the aggregated hemocytes appear to destroy and phagocytose the neuron to which they were attached (Farr et al. 2001). We observed amoebocytes similar to hemocytes that formed aggregates, and they were especially prominent in sheath cultures. In addition, we observed aggregates of amoebocytes around the tips of growing axons in sheath cell and neuron co-cultures; however, we never observed the destruction of neurons by attached amoebocytes (Kirk, unpublished observations). The latter result suggests that the majority of amoebocytes in our cultures were not hemocytes.

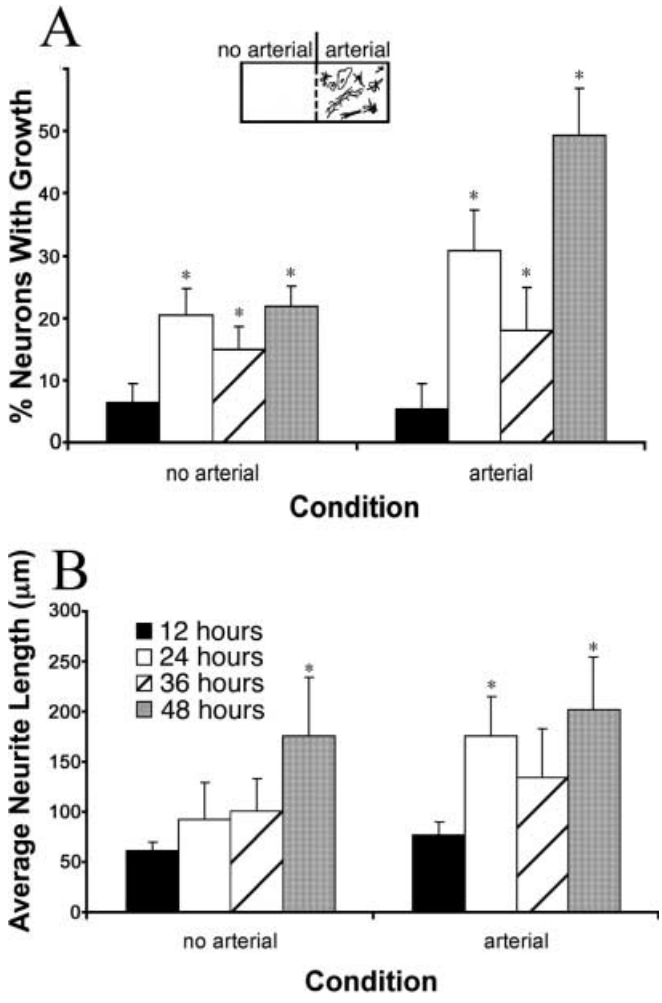

Fig. 10 Factor(s) produced by arterial cells significantly increased the percentage of neurons with growth (**A**) and the average neurite length per neuron (**B**), regardless of whether the neurons were in direct contact with arterial cells. The percentage of neurons with growth was increased throughout the coverslip (on both "arterial" and "no arterial" coverslip sides, see *inset*) at 24, 36, and 48 h post-plating of the neurons. The average neurite length per neuron was significantly enhanced at 48 h on the half of the coverslip lacking arterial cells (i.e., no arterial). The average length of neurite per neuron increased with time post-plating for neurons in contact with arterial cells (i.e., arterial) and the increase was significantly different ($P < 0.01$; *asterisks*) from controls at 24 and 48 h

We observed natural variability in the growth response by dispersed neurons to the various culture conditions. Some of the variability in neurite growth likely represents variations in the properties of sheath or arterial cells as well as the co-cultured neurons after isolation and plating of the cells. For instance, the dispersed cultures of pedal-pleural neurons represent a mixture of neuronal types. The enzymatic and mechanical methods to dissociate the neurons from the pedal and pleural ganglia are selective for smaller cells present in these central ganglia (Goldberg and Schacher 1998); even so, our neural cultures likely contained a mixture of sensory neurons, motor neurons (Ambron et al. 1996), and interneurons (Diaz-Rios et al. 1999). Therefore, some variability in neurite outgrowth may be due to differing responses by the various neural cell types included in our studies. Gruenbaum and Carew (1999) observed a similar degree of variability in neurite outgrowth by *Aplysia* bag cell neurons (a homogeneous population of neurons) grown in the presence of various vertebrate ECM substrates and growth factors.

Important issues to address in future experiments include: determining which cell type(s) in sheath and arterial cell cultures produce the growth factors, identifying and characterizing the molecular nature of the growth factors, determining the receptor and signaling pathways they use to enhance neurite growth, and testing whether and how these factors act during adult neural plasticity, such as in regeneration, learning, and memory.

Sheath and arterial cells as potential sources of growth factors

Large myocytes of the AA provide structural integrity to the aorta as well as the means for blood vessels to regulate blood flow (Alevizos et al. 1989). The average widths and lengths of AA myocytes in cell culture approach those observed in histological sections of the intact AA (Price et al. 1984). The smaller myocytes found in nerve sheath, including those of the PVCs, contract (in response to motor neuron input) and relax to determine the length of connectives (and peripheral nerves) as the animal alters its body dimensions, for example during locomotion (Umitsu et al. 1987).

In the arterial cultures, the myocytes are the largest, most prominent cell type in cultures of dispersed AA. The soluble factor(s) produced in arterial cell cultures can diffuse within the culture dish and act at a distance to enhance neurite growth. One possibility is that the diffusible factor produced in arterial cell cultures is a soluble form of acetylcholinesterase, which is present in *Aplysia* hemolymph and enhances neurite growth from isolated *Aplysia* neurons (Srivatsan and Peretz 1997). It is also possible to establish a substrate-bound form of growth factor(s) from *Aplysia* hemolymph (Burmeister et al. 1991), and it may be that the soluble form of acetylcholinesterase binds to coated glass coverslips and can promote neurite outgrowth. Given their large size and abundance, the source of the secreted soluble factor(s), in our cultures as well as in hemolymph, may be the arterial myocytes. Both membrane-bound and soluble forms of acetylcholinesterase enhance neurite growth and synapse development in vertebrates (Sternfeld et al. 1998). It is possible that soluble growth factors secreted into the hemolymph are needed to promote neurogenesis in *Aplysia*, as neurons continue to be added to the CNS throughout at least the juvenile stage of development (Cash and Carew 1989).

The regenerating neurites in our co-cultures were not overtly attracted to a particular sheath or arterial cell type. Therefore, it remains to be determined whether

more than one type of cell in these cultures secretes growth factors, and if the growth factors are produced by only one cell type, which cell type it is. Since both myocytes and fibrocytes are especially abundant in the sheath cell cultures, it appears likely that one or both produce the substrate-bound factor(s). Potential substrate-bound factors produced by the cultured sheath cells include *Aplysia* collagen (Floyd et al. 1999) and laminin or fibronectin (Ren et al. 1997). Integrins typically recognize short linear amino acid sequences (for example, RGD-Arginine-Glycine-Aspartate) in extracellular matrix proteins. Wildering et al. (1998) show that outgrowth from *Lymnaea* neurons, in response to substrates comprised of vertebrate ECMs, is induced by both RGD-dependent and RGD-independent signaling mechanisms, presumably mediated by cell adhesion receptors belonging to the integrin family.

Fejtl and Carpenter (1995) reported enhanced neurite outgrowth in the presence of medium conditioned using central ganglia of *Aplysia*. Sheath cells are an unlikely source of growth factors present in the latter conditioned medium because our results show that a neurite growth-promoting factor(s) produced by sheath cells does not freely diffuse from its source in aqueous cultures. In future experiments it will be important to determine if medium conditioned by the growth of sheath or arterial cell cultures will promote neurite outgrowth in separate neural cultures. Conditioned medium derived from the isolated CNS of *Lymnaea* has potent neurite growth-promoting activity (Woodin et al. 2002), and it is possible that arterial and/or CNS sheath cells produce the factors responsible for this neurotrophic activity.

In preliminary experiments, we killed sheath or arterial cells by exposure to air, rinsed debris from the coverslips, and grew neurons on the remaining substrate. Neurons grown on substrate remaining after sheath or arterial cells were killed showed significantly enhanced neurite growth (Vanmali, Messner, and Kirk, unpublished observations), indicating that both sheath cells and arterial cells produce factors that adhere to the coverslip and possess growth-promoting activity. Therefore, sheath cells at least produce a substrate-bound growth factor while arterial cells appear to produce both diffusible and substrate-bound growth factors. We have yet to test whether the sheath or arterial factors can increase the survival time of co-cultured neurons, a property exhibited by neurotrophic factors on select target neurons (Huang and Reichardt 2001; McKay et al. 1999).

In separate preliminary experiments, sheath cell cultures were labeled with an antibody for the presence of Ag, a secreted protein made by *Aplysia* glial cells. When *Aplysia* neurons are dissociated for cell culture, some Ag-positive glia remain attached to isolated somata (Lockhart et al. 1996). It is possible that some adaxonal glia expressing Ag were dissociated from the axonal core of the PVCs during our trituration protocol. For example, in sheath cell cultures we observed very small cells labeled for Ag. These cells are likely to be adaxonal glia, since these glia (found in the core of central connectives) are labeled with the Ag antibody (Lockhart et al. 1996). These cells were present in our control neural co-cultures (i.e., SL-15 alone) and, as mentioned above, these cells remain attached to *Aplysia* neural cell bodies after isolation. There was very limited neurite growth in control cultures, therefore, if Ag acts as a growth factor, it must be produced at limiting concentrations in culture conditions.

Neurotrophins in mollusks and other invertebrates

Although some invertebrate molecules are homologues of vertebrate growth factors (McKay et al. 1999; Muskavitch and Hoffmann 1990), to date no invertebrate neurotrophins have been identified, and neurotrophin and Trk receptor homologues are completely lacking in *C. elegans* and *Drosophila* (Adams et al. 2000; Jaaro et al. 2001; Ruvkun and Hobert 1998). However, homologues of vertebrate Trk receptors are present in mollusks (van Kesteren et al. 1998; McKay et al. 1999), and recently, a homologue to vertebrate Trks was discovered in *Aplysia* (Giustetto et al. 2000). Based on potential functional protein homology at the level of three-dimensional structure, McKay et al. (1999) suggest that cysteine-rich neurotrophic factor (Fainzilber et al. 1996), EGF (Hermann et al. 2000), and/or an insulin-related peptide (Smit et al. 1998), all characterized in *Lymnaea*, may be ligands for molluscan neurotrophin receptors. Thus, it appears that neurotrophin receptors, and their as yet unidentified ligands, exist in mollusks (including *Aplysia*) and may play important roles in neural plasticity (Gruenbaum and Carew 1999; McKay et al. 1999).

Potential roles for growth factors in long-term plasticity in adult organisms

In the adult CNS, soluble and substrate-bound growth factors influence neural regeneration (Fawcett and Asher 1999; Fournier and Strittmatter 2001; Moffett 1996) and synaptic plasticity, such as occurs during learning and memory (Bailey and Kandel 1993; McKay et al. 1999). In the vertebrate peripheral nervous system where regeneration is robust, Schwann cells phagocytose nerve debris in response to nerve injury, secrete neurotrophic factors, and produce ECMs that support axonal regeneration (Jones et al. 2001). When Schwann cells are grafted into the injured spinal cord, they promote axonal regeneration across the lesion site, especially when combined with growth factors (for example, BDNF and NT-3) secreted naturally by the Schwann cells in response to injury, administered exogenously, or released from genetically modified Schwann cells after transplantation. In some cases the enhanced axonal growth promoted by Schwann cells transplanted into the CNS after injury is associated with behavioral recovery (Blits et al. 2000; Jones et al. 2001).

In invertebrates like leeches and mollusks, microglial cells migrate to sites of injury in the CNS and appear to produce laminin (von Bernhardi and Muller 1996; Moffett 1996). In the case of lesions to the leech CNS, this accumulation of a laminin-like molecule is associated with extensive axonal sprouting and likely contributes to the outgrowth of neurites at the site of injury (Masuda-Nakagawa and Nicholls 1991; Masuda-Nakagawa et al. 1993, 1994). By analogy, growth factors such as laminin potentially produced by sheath cells in *Aplysia*, including microglia and/or adaxonal glia, may promote axonal regeneration in adult animals. For instance, sheath cells present at sites of crush lesions to CBCs in *Aplysia,* and/or microglia that migrate to the sites of a CBC crush lesion (Kirk, unpublished observations), could contribute to the axonal regeneration and functional recovery of feeding behavior observed in adult animals (Johnson et al. 1999; Scott et al. 1997b).

There is also a growing body of evidence that growth factors and ECMs contribute to long-term potentiation (LTP) in adult hippocampus, an intensively studied model for information storage during learning and memory in vertebrates. For instance, evidence supports laminin's role in the maintenance of LTP (Madani et al. 1999; Nakagami et al. 2000; Staubli et al. 1998). In addition, growth factors including cytokines and neurotrophins regulate activity-dependent synaptic transmission in adult organisms, including LTP (McAllister et al. 1999; Minichiello et al. 1999) and long-term depression (Akaneya et al. 1996).

Growth factors produced by *Aplysia* sheath and arterial cells may also influence other forms of adult neural plasticity such as long-term forms of synaptic plasticity that underlie learning and memory (McKay et al. 1999). *Aplysia* cell adhesion molecules (apCAMs) have been characterized and downregulation of apCAM expression on the surface of sensory neurons leads to neurite outgrowth and formation of new varicosities (i.e., sites of synaptic contact) during long-term facilitation (LTF) induced by serotonin (5-HT) (Bailey et al. 1997; Mayford et al. 1992). Hatada et al. (2000) provided evidence suggesting that loss of apCAMs at sites other than preexisting varicosities may be the initial trigger for growth of sensory neuron presynaptic processes in response to 5-HT.

Recent studies have implicated soluble growth factors in the induction of LTF in *Aplysia*. Zhang et al. (1997) reported that human recombinant TGF-β1 elicited LTF at sensorimotor synapses, and this effect did not summate with 5-HT induction of LTF, suggesting that TGF-β1 mediates the effects of 5-HT. In addition, they showed that a soluble portion of human TGF-β1 type II receptor (TβR-II) blocked the 5-HT-induced LTF, presumably by scavenging an endogenous form of TGF-β1. Thus, an *Aplysia* TGF-β1 appears to be both necessary and sufficient to induce LTF. Exogenous human BDNF can also produce LTF in *Aplysia* sensory to motor neuron synapses (McKay et al. 1999).

Chin et al. (1999) demonstrated the presence of TβR-II-like immunoreactivity in the *Aplysia* CNS, indicative of a putative receptor for an endogenous form of *Aplysia* TGF-β1. The TβR-II-like protein of *Aplysia* sensory neurons could bind an endogenous TGF-β1 and thus mediate the increased excitability and synaptic strength that accompanies LTF induced by 5-HT treatment. This type of long-term neural plasticity is correlated with increased sensory neuron branching and the formation of new varicosities between sensory and motor neurons (Bailey and Kandel 1993). Liu et al. (1997) hypothesized that LTF is in part mediated by apTBL-1, an *Aplysia* metalloprotease, that is secreted by sensory neurons in response to 5-HT. The apTBL-1 may subsequently activate an endogenous TGF-β1 or cleave procollagen to make an active form of collagen (Liu et al. 1997). An *Aplysia* homologue of vertebrate collagen has been isolated and characterized (Floyd et al. 1999), and generation of TGF-β1 and/or collagen may promote the neurite growth and increased synaptic contacts formed during LTF. It is possible that *Aplysia* collagen and/or a TGF-β1-like molecule is produced by the sheath and/or arterial cells in the co-cultures described here and are in part responsible for the enhanced neurite growth observed.

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